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13. ABSTRACT *(Maximum 200 words)*

This report summarizes studies on the mechanisms and treatment of soman-induced seizures and neuropathology. Our previous contract work showed that piriform cortex (PC) is the earliest cortical area involved in soman-induced seizures. Work in our laboratory and others indicate that seizure activity is maintained by NMDA receptors (NRs). Recent studies have shown that NRs exhibit varying subunit composition and the specific subunit composition determines the functional properties of NRs. The diversity of NR subtypes poses a serious challenge to the development of specific pharmacological interventions to block soman induced seizures. Ultimately, protective strategies will benefit from a description of the NR makeup of PC neurons. Thus, characterization of the composition of NRs in PC, a major goal of this contract, is an important step in the understanding and treatment of soman-induced seizures. This report summarizes the results of experiments using reverse transcriptase-polymerase chain reaction (RT-PCR) techniques to compare the relative levels of NR subunits in the PC and other cortical regions. The comparative expressions of NR1, NR2A, NR2B, NR2C, NR2D and NR3 subunits were analyzed in the PC, entorhinal, visual and motor cortices, and the olfactory bulb. This analysis showed that NR2B was the most heavily expressed subunit in all of the brain regions examined. These results suggest that NR2B is an important component of the NRs while NR2C, NR2D and NR-L are not essential for activities of most NRs in the forebrain areas examined. Strategies aimed at blocking seizures may need to focus on selective antagonists to the NR2B subunit. This report also describes a novel, long variant of the NR3 subunit identified in this contract work.

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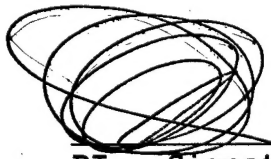

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CHAPTER I

INTRODUCTION

We have shown that piriform and entorhinal cortices are the earliest cortical areas involved in soman-induced seizures (Zimmer et al., 1977a,b, 1988). Experiments in our laboratory also indicate that the initial triggers for these soman-induced seizures are excess acetylcholine and norepinephrine (El-Etri et al., 1992; Ennis and Shipley, 1992; Zimmer et al., 1977a,b, 1988).. By contrast, the maintenance of seizure activity is mediated by activation of glutamate receptors, in particular, NMDA receptors. Administration of NMDA receptor antagonists block the induction of Fos in piriform cortex (PC), as well as convulsions, elicited by soman (Braitman and Sparenborg, 1989).

Recent studies have shown that the subunit composition determines the functional properties (open time, sensitivity to extracellular magnesium) of NMDA receptors. Thus, characterization of the composition of NMDA receptors in piriform and entorhinal cortices is an important step in the understanding and treatment of soman-induced seizures. A major goal of our contract work, therefore, was to characterize the expression of NMDA receptors in PC, entorhinal cortex, as other cortical structures. Chapter II or this report summarizes the results of experiments using reverse transcriptase-polymerase chain reaction (RT-PCR) techniques to compare the relative levels of NMDA receptor subunits in the PC and other cortical regions. The comparative expressions of NR1, NR2A, NR2B, NR2C, NR2D and NR-like (NR-L) subunits of the NMDA receptor were analyzed in the PC, entorhinal, visual and motor cortices as well as in the olfactory bulb of the adult rat. The analyses showed that NR2B was the most heavily expressed subunit in all of the brain regions examined. The expression levels of the NR1 and NR2A subunits were 50-70% of NR2B. NR2C, NR2D and NR-L mRNAs were present at very low

levels of only 2-7% of NR2B. Moreover, the motor cortex was totally lacking NR2D and NR-L messages. These results suggest that NR2B is an important component of the native NMDA receptors while NR2C, NR2D and NR-L are not essential for activities of most NMDA receptors in the forebrain areas examined.

In the course of the work described above, we discovered a new variant of NR-L (or NR 3) subunit of the NMDA receptor. In Chapter III, we analyzed the composition, and regional and developmental expression, of this new NR 3 NMDA receptor subunit variant. This work indicates that a longer variant of rat mRNA encoding the NR3 subunit of the NMDA receptor contains a 60-bp insertion at the nucleotide position 3007 in the intracellular domain of the C-terminal of the previously-cloned variant. This suggests that the NR3 mRNA exists in at least two variants – one with the insert (NR3-long; NR3-l) and one without the insert (NR3-short; NR3-s). The deduced 20 amino acid residues of the insert revealed two potential phosphorylation sites which may play a role in regulating functional properties the NR3-l subunit. The NR3-l variant is expressed throughout the adult rat brain. Moreover, this variant predominates in the occipital and entorhinal cortices, thalamus and cerebellum. Analysis of NR3-l development indicates that it is regulated in a region-specific manner.

CHAPTER II

Expression of NR1, NR2A-D and NR3 Subunits of the NMDA Receptor in Five Regions of the Rat Forebrain

INTRODUCTION

NMDA receptors participate in a wide variety of normal and pathological processes in the vertebrate nervous system. In particular, they play an important role in long-term potentiation (LTP) that is involved in memory formation and learning (Artola and Singer, 1994; Sakimura et al., 1995). Excessive NMDA receptor activation is thought to be critical to such pathological conditions as seizures and neuronal cell death following ischemia, hypoglycemia, HIV infection, and head trauma (Choi, 1988 and 1992; Meldrum and Garthwaite, 1990; Lipton and Rosenberg, 1994).

The genes encoding the subunits of the NMDA receptor have been identified and classified as three related families, NR1, NR2 and NR3 (the latter also known as NR3A, NR-like and χ-1 (Moriyoshi et al., 1991; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992.; Yamazaki et al., 1992; Ishii et al., 1993; Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998). The NR1 family consists of a single member, while the NR2 family includes NR2A, NR2B, NR2C and NR2D subunits (Sprengel and Seeburg, 1994). The NR3 family has recently been identified. So far, only one member of this family has been cloned (Ciabarra et al., 1995; Sucher et al., 1995).

Heterologous co-expression studies in mammalian cells demonstrated that formation of NMDA channels require a combination of the NR1 subunit and at least one of the NR2 subunits (Boeckman and Aizenman, 1994; Stern et al., 1994; McIlhinney et al., 1996). The co-expression studies have also demonstrated that many biophysical and pharmacological properties of the heteromeric NR1/NR2 NMDA receptor channels, such as sensitivity to

Mg^{2+} block, kinetics of desensitization and offset decay, susceptibility to modulation by glycine, reducing agents, polyamines and phosphorylation, as well as affinity for agonists and antagonists, depend on the type of NR2 subunit included in a heteromeric complex (Moriyoshi et al., 1991; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992, 1994; Buller et al., 1994; Brimecombe et al., 1997; Vicini et al., 1998). The NR3 subunit also does not by itself generate agonist-activated currents. However, it decreases such current when co-transfected with the NR1 and NR2 subunits into *Xenopus* oocytes (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998).

Studies of the native NMDA receptors in the brain using *in situ* hybridization and immunocytochemical analysis revealed a wide-spread presence of the NR1 subunit with other subunits displaying varying but overlapping distributions (Moriyoshi et al., 1991; Monyer et al., 1992, Ishii et al, 1993; Buller et al., 1994). In addition, co-immunoprecipitation experiments revealed that NR1 subunits usually precipitated in a complex with other NMDA receptor subunits (Sheng et al., 1994; Chazot and Stephenson, 1997; Luo et al., 1997; Das et al., 1998). Therefore, the native NMDA receptors are likely to be a heterogeneous group consisting of NR1 subunit associated with various combinations of NR2 and NR3 subunits. The multiplicity of subunit combinations which may constitute the NMDA receptor complex has been postulated as a basis for functional and pharmacological diversity of this receptor in the brain and the spinal cord (reviewed in Sucher et al., 1996).

In contrast to numerous reports describing distributions of individual NMDA receptor subunits in the developing and mature central nervous system (Moriyoshi et al., 1991; Monyer et al., 1992, 1994; Ishii et al, 1993; Wang et al., 1995; Zhong et al., 1995; Dunah et al., 1996; Lin et al., 1996; Portera-Caillau et al., 1996; Standaert et al., 1996; Wenzel et al., 1996, 1997; Schito et al., 1997), there are virtually no studies directed at examination of the relative levels of the entire range of these subunits and their messages in most brain regions. This information, however is an essential step toward better understanding of the

native combinations of subunits underlying the known region-specific differences in functionality of the NMDA receptors (Buller et al., 1994; Monyer et al., 1994; Sucher et al., 1996). The literature search also revealed that the majority of the published studies have lumped the cytoachitectually and functionally diverse parts of the cerebral into a single item (Moriyoshi et al., 1991; Monyer et al., 1992, 1994; Ishii et al, 1993; Wang et al., 1995; Zhong et al., 1995; Dunah et al., 1996; Lin et al., 1996; Portera-Caillau et al., 1996; Standaert et al., 1996; Wenzel et al., 1996, 1997; Schito et al., 1997), rather then examining the NMDA receptor subunits in individual regions of the isocortex, allocortex and mesocortex.

In the present paper, we describe the analysis of the relative expression levels for the NR1, NR2A-D and NR3 subunits of the NMDA receptor in the piriform region of the allocortex, the entorhinal region of the mesocortex, the granular visual and agranular motor regions of the isocortex of the adult rat. The selected cortical areas represent phylogenetically and/or structurally and functionally distinct regions (Affifi and Bergman, 1998) which also differ in their propensity to support such NMDA-related activities as long-term potentiation (LTP) and seizures (Piredda and Gale, 1985, 1986; McIntyre and Wong, 1986; Croucher et al., 1988; Racine et al., 1988; Stripling et al., 1988; Kanter and Harberly, 1990; Kimura et al., 1989; Artola and Singer, 1994; Halonen et al., 1994; Araki et al., 1995; Castro-Alamancos et al., 1995; Castro-Alamancos and Connors, 1996; Kudoh and Shibuki, 1997).We also examined the relative levels of the NMDA receptor subunits in the adult olfactory bulb. The latter structure was included in the study as the only region retaining many features of the immature nervous system in adult mammalian brain (Farbman, 1994; Doetsch et al., 1997; Goldman, 1998). A significant developmental regulation of the NMDA receptor subunits is well documented (Monyer et al., 1994; Williams et al., 1993; Riva et al., 1994; Wang et al., 1995; Zhong et al., 1995; Dunah et al., 1996; Portera-Caillau et al., 1996; Wenzel et al., 1996, 1997). Therefore, it is of interest to determine whether olfactory bulb has the relative levels of expression of the

NMDA subunit significantly different from other conventionally mature regions examined. The levels of the relevant transcripts in the brain tissue was assayed by the reverse transcriptase - polymerase chain reaction (RT-PCR) which allows detection and quantification of low amounts of specific mRNAs in small tissue samples.

MATERIALS AND METHODS

Six adult male rats were anesthetized with halothane and decapitated. Brains were taken out rapidly. The piriform, entorhinal, visual, and motor cortices and the olfactory bulbs were dissected out and immersed in liquid nitrogen and then stored at -70°C for subsequent extraction of RNA. The cortical regions were defined according to Paxinos and Watson (1997). The motor region included the M1 and M2 areas and the visual region included the V1M, V1b and V2 areas.

Isolation of Total RNA and Reverse Transcription of cDNA. Total RNA from samples of individual brain regions was isolated with the RNeasy columns using the protocol recommended by the manufacturer (QIAGEN, Chatsworth, VA). The RT reactions were carried out at 37°C for 60 min. Each reaction included 10 μg of denatured total RNA in a volume of 40 μl containing 300 ng of hexamer random primers (Gibco BRL, Gaithersburg, MD) and first-strand reaction mix beads, incorporating Moloney Murine Leukemia Virus reverse transcriptase, buffer, dNTP, RNAguard and RNase/DNase-free BSA (Pharmacia, Piscataway, NJ). The reactions were stopped by heating at 70°C for 15 min. The concentration of cDNA was assessed with the Hitachi U-1100 Spectrophotometer (Hitachi, Tokyo, Japan) using a nucleotide absorption at 260 nm.

Oligonucleotides Used for cDNA Amplification. NMDA-receptor subunit-specific primers were: CTGCAACCCTCACTTTTGAG/TGCAAAAGCCAGCTGCATCT which amplified the 145 base pairs (bp) 2910-3055 sequence of the cDNA common to all splice variants of the rat NR1 subunit of the NMDA receptor; GACGGTCTTGGGATCTTAAC/TGACCATGAATTGGTGCAGG which amplified the

140 bp 957-1240 sequence of the cDNA of the rat NR2A subunit; CAAGAACATGGCCAACCTGT/GGTACACATTGCTGTCCTTC which amplified the 229 bp 2772-3001 sequence of the cDNA of the rat NR2B subunit; TGGAAACTTCGACACTCGGT/TCCAAAGAGCTGCTCACGTC which amplified the 220 bp 2541-2761 sequence of the cDNA of the rat NR2C subunit; AGCGATAGCCGACGGCTTCCA/ACGATGGCGAAGTAGGAAGGT which amplified the 162 bp 3594-3756 sequence of the cDNA of the rat NR2D subunit; ACATAGTGCACAGACTGCTG/CGTTGGTTGTCATGACTCAG which amplified the 219 bp 2911-3073 sequence of the cDNA of the rat NR3 subunit. We also employed primers CATCCTGAAGCATACAGGTC/CAGAAGGAATGGTTTGATGG amplifying the 257 bp 306-563 sequence of the cDNA of the cytoplasmic protein cyclophylin which we used for normalization of the expressions of different NMDA receptor subunits in each brain area examined (see below). The BLASTN program (NCBI, Bethesda, MD) was used to ensure that all amplified sequences shared no homology and were different from any other cDNA in the data-base (Altschul et al., 1990).

Polymerase Chain Reaction. PCR was performed in a mixture containing 50 ng of cDNA reversely transcribed from the total sample RNA (RT-cDNA), 0.25 μM of each of the 5' and 3' sequence-specific primers and a PCR bead (Pharmacia, Piscataway, NJ). When brought to a final volume of 50 μl, each reaction included 3 units of *Taq* polymerase, 10 mM Tris-HCl, (pH 9.0 at room temperature), 50 mM KCl, and 200 mM of each of the dNTPs. The solution also incorporated 1.5-2.5 mM $MgCl_2$ depending on the specific reaction. The amplification was performed on a DeltaCycler System (ERICOMP, San Diego, CA), with denaturation at 94 °C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The final cycle was followed by a 10 min extension step at 72°C to ensure that the amplified DNA was double stranded. For all samples, 28 amplification cycles were performed. We found these conditions appropriate for every PCR used in this study.

Analysis of the Amplified Products. Fifteen μl of each amplified sample was run in parallel with a DNA ladder (Gibco BRL. Gaithersburg, MD) on a 2% ethidium bromide-stained agarose gel. After electrophoresis, the gel was illuminated with UV light and digitized with Panasonic WV-BP312 B/W camera (Yokahama, Japan) and Oncor Imaging system (Pittsburgh, PA). The digitized images were analysis with the Universal Software for Electrophoresis & TLC (Advanced American Biotechnology, Fullerton, CA). The number of fentomoles of the amplified product in each sample was estimated by comparing the intensity of the ethidium bromide staining produced by the PCR product band with that of six known amounts of the same product run on the same gel and serving as quantification standards.

The specificity of the amplification was confirmed by comparison of the PCR products from all tissue samples with the products obtained from the plasmids containing cDNA of the individual NMDA receptor subunits as well as the cyclophilin cDNA. The images generated by electrophoresis on agarose gel revealed that the molecular weights of all the fragments amplified by each sets of primers were identical independently on whether the original source was the tissue RT-cDNA or specific cDNA-containing plasmids (Fig. 1). Furthermore, all the amplified fragments had the predicted size (Fig. 1).

To assure the quantitative nature of our PCR analysis, we also examined the quantities of PCR product (in fentomols) produced by each set of primers from increasing amounts of RT-cDNA (in nanograms) generated from all forebrain regions studied, with the amounts of the cDNA subjected to amplification spanning over at least two orders of magnitude. The use of the RT-cDNA, rather than the total tissue RNA, allowed us to avoid possible problems with variations in the efficiency between individual RT reactions and is fully justified under a reasonable assumption that the inter-reaction variability in the reverse transcription efficiency have equal effects on the yield of all the cDNA species in a given sample. The statistical analysis (this and all other statistics were performed with Prism software, Graph Pad, San Diego, CA) showed that all the resultant data can be best fitted

by the logarithmic regression curves and that the amplification was below saturation levels for all the amounts of the RT-cDNA tested with each set of primers. The latter conclusion can be clearly drown from the linear relationship between the logarithms of the initial RT-cDNA amounts and the quantities of the amplification products seen in Figure 2. This indicates that the amplification protocols employed in our study are appropriate for quantitative analysis as long as the amount of the RT-cDNA used is within the range tested above (Kohler, 1995). In all our amplification assays we used 50 ng of the RT-cDNA, which is well within the appropriate range.

In addition to examining the quantities of the PCR products obtained from increasing amounts of the RT-cDNA, we analyzed the quantities of the amplification products (in fentomoles) generated by each set of primers from various amounts of plasmids containing cDNAs of individual NMDA receptor subunits as well as cyclophylin cDNA (in attomols). Here, once again, the data are best fitted by the logarithmic regression with the relationship between the initial amount of plasmids and the quantities of amplification product becoming linear when plotted with X-axis on the logarithmic scale (Fig, 2). The Tukey's analysis of the slopes of the regression lines (based on the 95% confidence intervals) for the amplification products produced by the same primers from the plasmid cDNA and from the RT-cDNA (Fig. 2) showed lack of statistical differences ($p > 0.05$) suggesting that these regression lines are parallel. This indicates that the regressions representing the relationship between the amounts of the specific cDNA-containing plasmids in the PCR mixture and the quantities of the resultant PCR product can be used for comparative analysis of the efficiencies of amplification of the same products from the RT-cDNA (Kohler, 1995).

The two-way ANOVA of the slopes and Y-axis intercepts (based on the 95% confidence intervals) of the regression lines representing the relationship between the amounts of the plasmids containing different specific cDNAs and the resultant amplification products (Fig, 2) showed these lines to be neither overlapping no parallel to each other ($p < 0.05$ for both slopes and Y-axis intercepts). This points to significant differences in the

efficiencies of amplifications with different primers employed in our assays. To compensate for these differences, this study utilized the equations of the aforementioned regressions representing the relationship between the amounts of the plasmids containing different specific cDNAs and the quantity of the products produced by amplification of these amounts to convert the fentomoles of each product into the attamoles of the initial cDNA molecules in the PCR mixture carrying the amplified sequences. The resultant numbers, rather than directly-obtained quantities of each PCR product, were used in further analysis (Kohler, 1995).

The final output for each receptor subunit was expressed as the attamoles of its cDNA per attamoles of the cyclophylin cDNA in the same amount of the sample RT-cDNA. This normalization allowed comparison of the expression levels of different NMDA receptor subunit in the same brain region but not between the regions since the cyclophylin messages are known to be subjects of region-specific regulation (Kohler, 1995). We would also like to note that, while PCR with the cyclophylin-specific primers was performed utilizing the same RT-cDNA samples which were used for PCR with the NMDA receptor subunit primers, these reactions were done in different tubes to avoid possible competition. To diminish the influence of the inter-tube variability of loading and amplification, the PCR with every set of primers was conducted 8 separate times for every forebrain area from each of the six animals examined in this study, which is a total of 48 repeats.

For the presentation of the data, the mean values ± SEM of all aforementioned repeats were calculated. In addition, for each brain area, the data representing the levels of expression of the individual NMDA receptor subunits were examined with one-way ANOVA followed by the Tukey's post-hoc analysis. $p < 0.05$ indicated statistically significant differences.

RESULTS

Piriform Cortex. We found that the piriform cortex expressed all six NMDA receptor subunits studied (Fig. 3). However, there were significant differences between the levels of their expression (Fig. 3). NR1 was clearly the predominant transcript. The expression of NR2A was only slightly higher than half of that of NR1. NR2B was expressed even at lower levels (~ 50% of NR2A). NR2C and NR3 were expressed at similar levels which were approximately 50% of those of NR2B. NR2D had the lowest levels of expression (~ 15% of NR2C/NR3).

Entorhinal Cortex. The entorhinal cortex also contained messages for all six NMDA receptor subunits (Fig. 3). In this region, however, NR1, NR2A and NR2B subunits were expressed at statistically similar levels (Fig. 3). The other three subunits were expressed at much lower levels (Fig. 3). The expression of NR3 was only around 14% of that of NR1/NR2A/NR2B, with NR2C and NR2D being expressed at 50% and 10% of that of NR3 respectively.

Visual Cortex. The visual cortex was similar to entorhinal region in that NR1, NR2A and NR2B were clearly the predominant transcripts with the expressions for the other three NMDA receptor subunits being significantly lower (Fig. 3). Among the latter transcripts, NR2C and NR3 were expressed at similar levels equal to approximately 25% of those of NR1/NR2A/NR2B. As in the case of the piriform and entorhinal regions, NR2D was the least abundant transcript in the visual cortex, being expressed only at less than 6% of that of NR2C/NR3 (Fig. 3).

Motor Cortex. The relative expression levels of the NMDA receptor subunits in the motor cortex was significantly different from those seen in the previously-described cortical regions (Fig. 3). This was the only cortical region examined where NR2B was clearly the predominant transcript (Fig. 3). The NR1 subunit was expressed at levels less that 75% of those of NR2B. NR2A had even lower levels of expression, approximately 50% of those

of NR1. The NR2C expression was half of that of NR2A. Another unique feature of the motor cortex was an apparent absence of the NR2D and NR3 transcripts (Fig. 3).

Olfactory Bulb. The final structure examined in this study, the olfactory bulb, expressed all six NMDA receptor subunits examined in this study (Fig. 3). The NR1 and NR2B were the most abundant transcripts, and were expressed at statistically similar levels (Fig. 3). The NR2A expression was close to 80% of that of NR1/NR2B. NR2C was expressed at levels of less that 20% of those of NR2A. The olfactory bulb was different from the cortical regions in that it contained a relatively high levels of the NR2D transcript (~ 75% of NR2C) whose expression was statistically similar to that of NR3 (Fig. 3).

DISCUSSION

The quantitative analysis presented in this paper provides full confirmation to previous, largely qualitative studies indicating that, among the NMDA receptor subunit transcripts, NR1, NR2A and NR2B are much more prevalent compared to NR2C, NR2D and NR3 in both the cerebral cortex and olfactory bulb of the adult rat (Moriyoshi et al., 1991; Kutsuwada et al., 1992; Meguro et al., 1992; Ishii et al., 1993; Buller et al., 1994; Monyer et al., 1994; Zong et al., 1995; Linden et al., 1997; Wenzel et al., 1997). This matches the predominance of the NR1, NR2A and NR2B proteins among the cortical and bulbar NMDA receptor subunit proteins (Wang et al., 1995; Dunah et al., 1996; Portera -Cailiau et al., 1996; Wenzel et al., 1996, 1997). In addition, our study demonstrates that a somewhat higher enrichment of NR2B protein as compared to NR2A protein, previously observed in the olfactory bulb (Portera-Cailliau et al., 1996; Wenzel et al., 1997), is well matched by slightly higher levels of expression of NR2B than NR2A messages in the same brain structure. The latter observation is particularly pertinent to the question of how close the measured tissue levels of the NMDA receptor subunit mRNAs mirror the levels of the corresponding proteins. It is well known that the quantity of a given mRNA in the tissue reflects not only the amount but also the efficiency of translation and the turnover rate of the

corresponding protein, and it is widely believed that only very large differences in the expression between the messages are indicative of the differences in the levels of the relevant proteins. However, the present results demonstrate that, al least in some cases, the relative proportions of the individual NMDA receptor subunit transcripts and proteins are very similar to each other. A rather close matching between the levels of the NMDA receptor subunit proteins and transcripts in multiple regions of the adult brain have also been reported by other investigators (Dunah et al., 1996; Wenzel et al., 1996; 1997). Therefore, it is reasonable to expect that the relative levels of messages encoding the individual NMDA receptor subunits observed in the present study are indicative of the relative proportions of the corresponding subunit proteins.

An important finding of our research is that individual regions of the rat cerebral cortex display fairly different relative levels of expression of the NMDA receptor subunits. In this respect, of particular interest is the motor cortex which differs from other regions examined by expressing virtually no NR2D and NR3 subunits of the NMDA receptor. Furthermore, this cortical areas was also unique in expressing the NR2B transcript at proportions significantly exceeding those of the NR1 and NR2A subunits of the NMDA receptor, with the latter message being present only at a third of the N2B levels. This observation is surprising, since the prevalence of the NR2B mRNA and protein is a characteristic of the developing, rather than the adult forebrain (Williams et al., 1993; Manyer et al., 1994; Zong et al., 1995; Portera-Cailliauet al., 1996; Wenzel et al., 1997), and the motor cortex retains neither structural nor functional characteristics of the immature nervous tissue (Jones and Peters, 1986).

We speculate that at least some of the detected motor cortical peculiarities in the relative expression of the NMDA receptor subunits may be related to an apparent low propensity of this region for generating NMDA receptor-dependent LTP and seizures (Baze, 1993; McDonough and Shih, 1993; Castro-Alamancos et al., 1995; Castro-Alamancos and Connors, 1996; Scremin et al., 1997; Zimmer et al., 1997a, 1997b). The aforementioned

functional trait of the motor cortex may be especially associated with a much higher expression of the NR2B NMDA receptor subunit as compared to NR2A subunits, since such significant prevalence of the NR2B transcript is not observed in the visual, entorhinal or piriform regions which are known to be common cortical sites for LTP and/or seizure generation (Piredda and Gale, 1985, 1986; McIntyre and Wong, 1986; Morgan et al., 1987; Croucher et al., 1988; Racine et al., 1988; Stripling et al., 1988; Alonso et al., 1990; Kanter and Harberly, 1990; Loscher and Ebert, 1996; Kudoh and Shibuki, 1997; Scremin et al., 1997; Zimmer et al., 1997a, 1997b; Wada et al., 1998). Furthermore, the piriform cortex, which is the region with the strongest potential for producing NMDA receptor-dependent LTP and seizures (Piredda and Gale, 1985, 1986; McIntyre and Wong, 1986; Morgan et al., 1987; Croucher et al., 1988; Racine et al., 1988; Stripling et al., 1988; Kanter and Harberly, 1990; Loscher and Ebert, 1996; Zimmer et al., 1997a, 1997b), has a much higher levels of the NR2A than NR2B transcript, which is completely opposite to what has been described above for the motor cortex.

In addition to displaying a significant predominance of the NR2A over NR2B transcripts, the piriform cortex is the only forebrain area examined where the expression of the NR1 subunit is much higher than those of either NR2A or NR2B subunits. In contrast, the visual and entorhinal cortices have relatively similar levels of the NR1, NR2A and NR2B messages. The functional implications of these observations are presently unknown.

As mentioned earlier, the olfactory bulb is the only mammalian brain structure which continues to incorporate newly-generated neurons and form new connections with the perpetually growing sensory innervation not only during development but also during the entire adult life (Farbman, 1994; Doetsch et al., 1997; Goldman, 1998). Not surprisingly, we found that the expression of the NMDA receptor subunits in this region retained some of the features characteristic of the immature brain. These features include a relatively high levels of the NR2D message and a slight predominance of the NR2B over NR2A transcript. Both of these subunits are over-expressed in the late fetal/early postnatal brain

(Williams et al., 1993; Manyer et al., 1994; Zong et al., 1995; Dunah et al., 1996; Portera-Cailliauet al., 1996; Wenzel et al., 1996) where their presence in the postsynaptic NMDA receptor complex results in lengthening of the duration of this receptor-mediated excitatory current (Ishii et al., 1993; Monyer et al., 1994; Wenzel et al., 1996; Li et al., 1998). These properties may facilitate coupling of asynchronous presynaptic activity and postsynaptic depolarization as is presumably the case in the developing nervous tissue (Monyer et al., 1994). It is tempting to suggest that the NR2B- and/or NR2D-containing NMDA receptors may play the same role in supporting functionality of continuously renewing neuronal connections in the adult olfactory bulb (Farbman, 1994).

FIGURE LEGENDS

Figure 1. Ethidium bromide-stained agarose gel showing PCR amplification products obtained with NR1-, NR2A-, NR2B-, NR2C-, NR2D- and NR3-specific primers. **P** - products of amplification of plasmids containing receptor subunit cDNA (positive control). **T** - products amplified from the cDNA reversely transcribed from the total mRNA of the piriform cortex. **m** - the molecular-weight marker. Note that the molecular weights of the fragments amplified from reversely transcribed cDNA are identical to those resulted from the plasmid amplification for all the subunit-specific primers. Similar results were also obtained for the cyclophylin primers (not shown).

Figure 2. Charts representing the relationship between the amounts of the plasmids containing the NMDA receptor subunit cDNAs (in attamoles) as well as the amounts of cDNA reversely transcribed from the total piriform cortical mRNA (in nanograms) in the PCR mixture and the resultant products (in fentomoles) produced by amplification with the NR1-, NR2A-D- and NR3-specific primers. The data are fitted with regression lines (Prism software, Graph Pad, San Diego, CA) which are linear when the X-axis is presented on the logarithmic scale. Note that for each subunit-specific primer, the

regression lines for the product generated by amplification of plasmids and for the product generated be amplification of the reversely transcribed cDNA are parallel. Also, the amplification is below the saturation levels for all the amounts of the reversely-transcribed cDNA tested. Similar charts have been obtained for the cyclophylin-specific PCR (not shown).

Figure 3. Bar graphs representing the relative expression levels of the NMDA receptor subunits in the piriform, entorhinal, visual and motor cortical areas and the olfactory bulb of adult rat. For each brain region, the columns represent the ratio of attomoles of cDNA of individual NMDA receptor subunits per attamoles of the cyclophylin cDNA in the same amount of the sample cDNA. The error bars are SEMs (n = 48). The lack of statistically significant differences is indicated by the "-" sign. The graphs can be used only for comparison of the expression levels of different NMDA receptor subunits only within each brain region, but not between the regions.

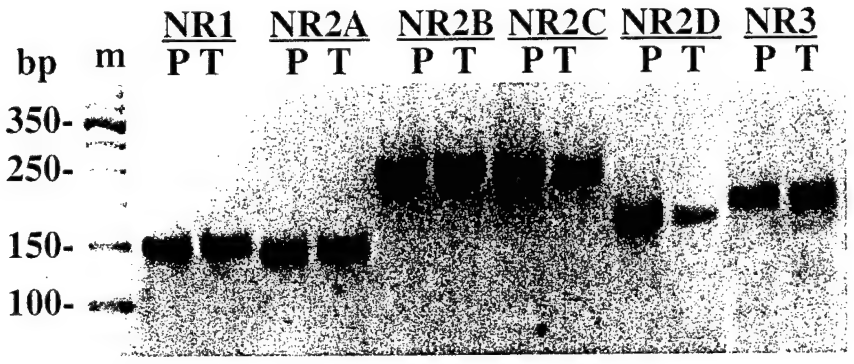

Figure 1

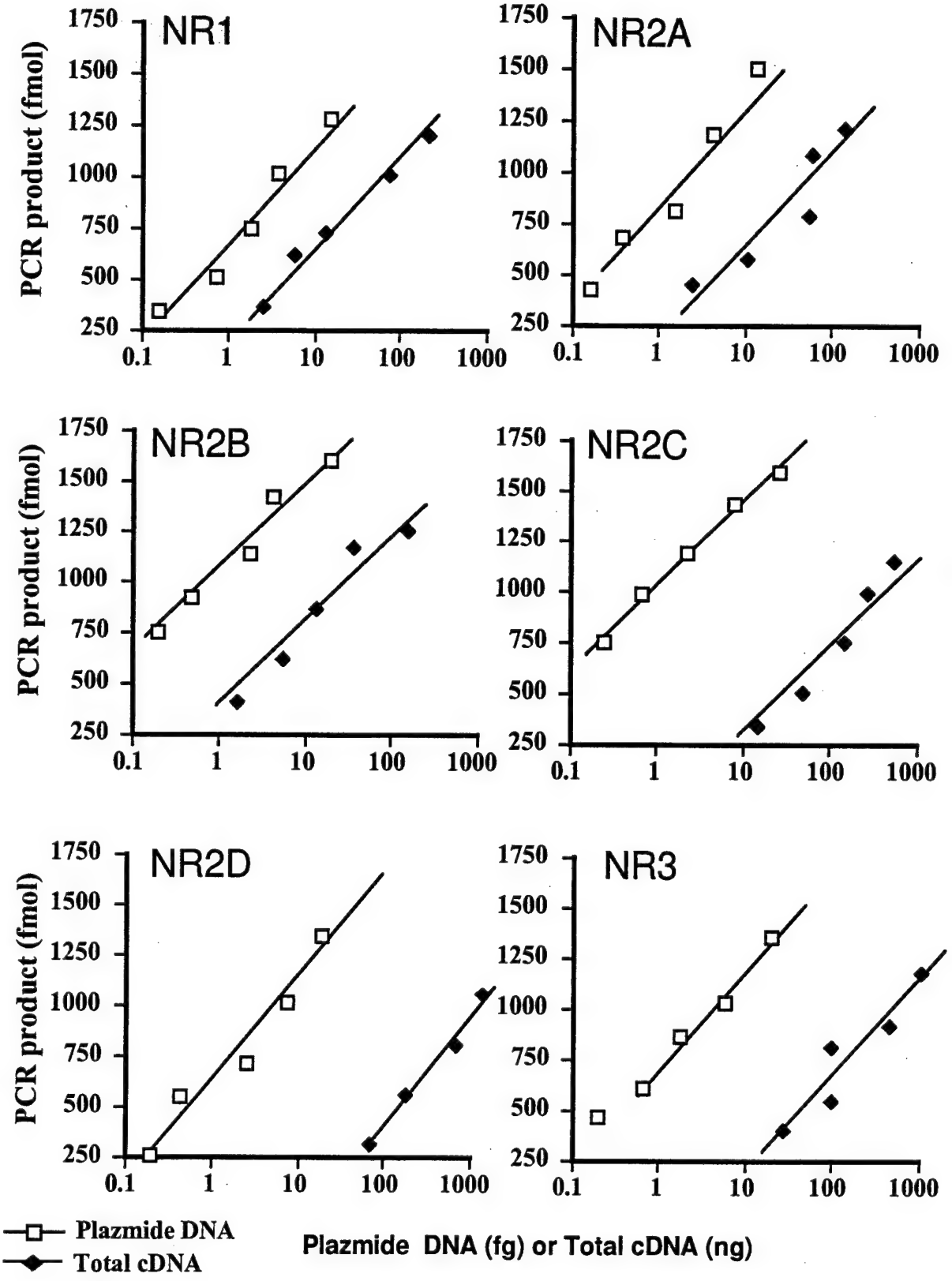
NR1
NR2A
NR2B
NR2C
NR2D
NR3
PCR product (fmol)
1750
1500
1250
1000
750
500
250
0.1
1
10
100
1000
Plazmide DNA
Total cDNA
Plazmide DNA (fg) or Total cDNA (ng)

Figure 2

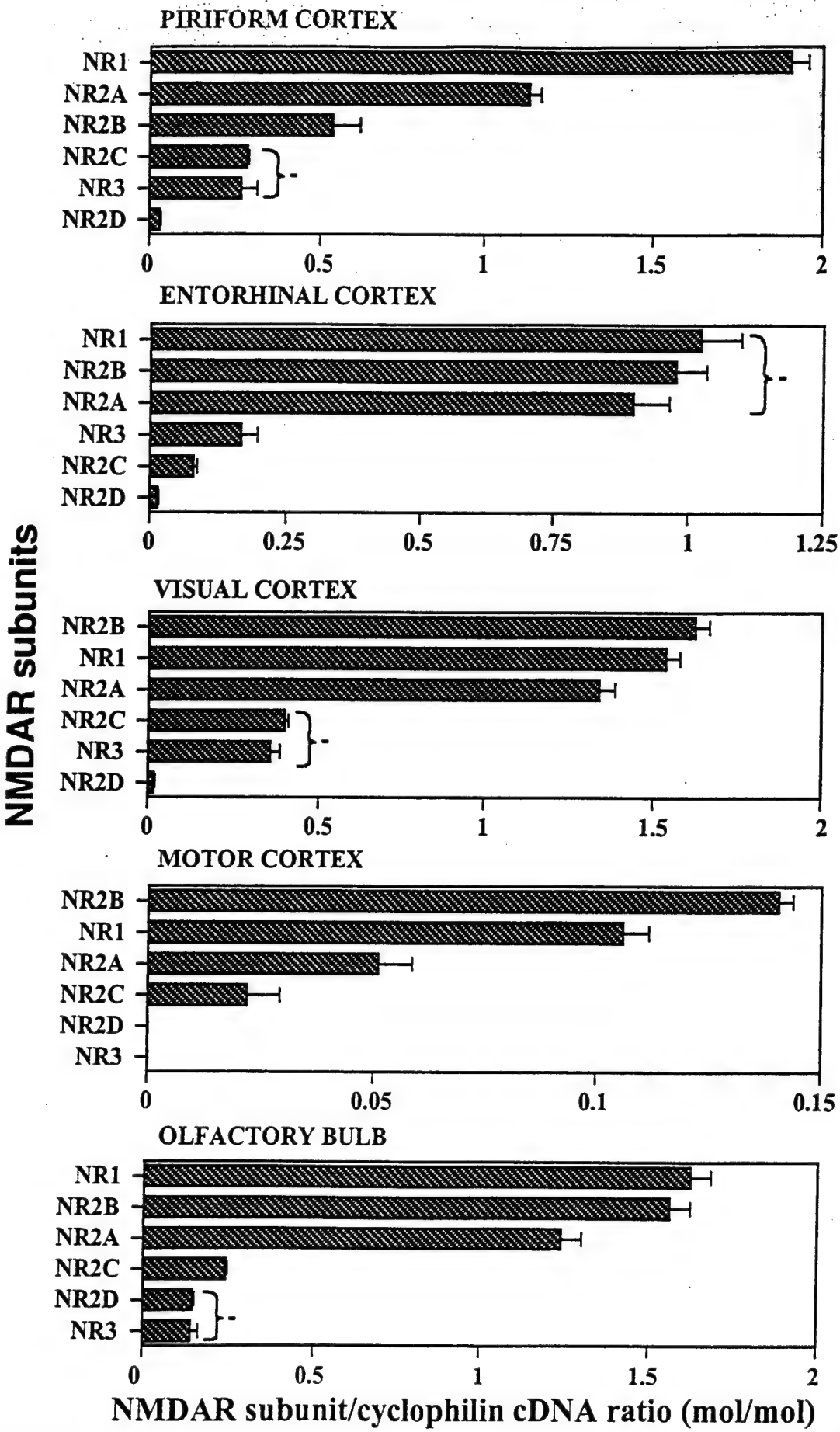
PIRIFORM CORTEX
NR1
NR2A
NR2B
NR2C
NR3
NR2D
0
0.5
1
1.5
2
ENTORHINAL CORTEX
NR1
NR2B
NR2A
NR3
NR2C
NR2D
0
0.25
0.5
0.75
1
1.25
VISUAL CORTEX
NR2B
NR1
NR2A
NR2C
NR3
NR2D
0
0.5
1
1.5
2
MOTOR CORTEX
NR2B
NR1
NR2A
NR2C
NR2D
NR3
0
0.05
0.1
0.15
OLFACTORY BULB
NR1
NR2B
NR2A
NR2C
NR2D
NR3
0
0.5
1
1.5
2
NMDAR subunits
NMDAR subunit/cyclophilin cDNA ratio (mol/mol)

Figure 3

CHAPTER III

Identification of a Long Variant of mRNA encoding the NR3 Subunit of the NMDA Receptor: Regional Distribution and Developmental Expression in the Rat Brain

INTRODUCTION

The NMDA receptors constitute a major class of the excitatory amino acid-gated ion channels. Molecular cloning efforts have identified six rat NMDA receptor subunits: NR1, NR2A, 2B, 2C and 2D (NR2A-D) and NR3 (the latter also known as NR3A, NR-like and χ-1); (Morioshi et al., 1991; Kutsuwada, et al., 1992; Meduro et al., 1992; Monyer et al., 1992; Watanabe et al., 1992; Ishii et al., 1993; Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998). Heterologous co-expression studies demonstrated that NR1 is an essential channel-forming NMDA receptor subunit. In contrast, the NR2A-D subunits do not by themselves produce functional channels but potentiate NR1 activity and confer functional variability to the NMDA receptors ((Morioshi et al., 1991; Kutsuwada, et al., 1992; Meduro et al., 1992; Monyer et al., 1992; Monyer et al., 1994). NR3 is also a regulatory subunit. Its presence decreases the channel currents in NR1/NR2 heteromeric receptors (; Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998). The existence of multiple NMDA receptors composed of different subunit combinations may underlay a great functional diversity characteristic of the native NMDA receptors.

The diversity of the NMDA receptors could be further enhanced by the alternative splicing of pre-mRNAs of receptor subunits. Until now, only the NR1 variants have been identified (Durand et al., 1992; Nakanishi et al., 1992; Kusiak and Norton, 1993; Sugihara et al., 1992). The eight known variants of this subunit display a high degree of functional heterogeneity. The homomeric NMDA receptors assembled from these NR1 splice variants

differ in agonist affinity, non-competitive antagonist affinity, current amplitude, pH sensitivity, Zn^{2+} modulation, polyamine potentiation, and regulation by protein kinase C (PKC) (Zheng et al., 1994; Durand et al., 1993; Hollmann et al., 1993; Zhang et al., 1994; Traynelis, et al, 1995).

In this paper, we report that mRNA encoding the NR3 subunit also exists as at least two forms. The new variant, which we named NR3-long (NR3-l), differs from the previously-described form by a 60-bp insertion within the intracellular domain of the C-terminal. The earlier cloned form of NR3 will now be referred to as NR3-short (NR3-s). We also examined the regional and developmental expressions of the new NR3 variant.

MATERIALS AND METHODS

RNA Extraction and cDNA Synthesis. Total RNA was extracted from the rat brain tissue using RNeasy columns (QIAGEN, Chatsworth, VA). Poly(A)$^+$ RNA was prepared employing a Poly(A)-pure mRNA Isolation Kit (Ambion Inc., Austin, TX). First strand complementary DNA (cDNA) synthesis was primed with random hexamers using First-strand beads (Pharmacia, Piscataway, NJ).

cDNA Cloning. Single-strand cDNA, reversely transcribed from rat telencephalon poly(A)$^+$ RNA, was used in the PCR reactions with two pairs of primers: (1) ACATAGTGCACAGACTGCTGTT (forward) and TCGTTGGTTGTCATGACTCA (reverse); (2) CTAGACGCCTTCATCATGGAC (forward) and CTCTGTCTTCCTGCTTACAGC (reverse). The first pair was designed based on the unique sequence of NR3, which spans the intracellular C-terminal region (Fig. 4A). The second pair amplifies the portion encompassing transmembrane regions III-IV (TMs III-IV) extracellular loop and the C-terminal (Fig. 4A). The above-mentioned PCR reactions were conducted on a DeltaCycler System (ERICOMP, San Diego, CA) using PCR beads (Pharmacia, Piscataway, NJ). Thirty two cycles of amplification were performed with denaturation at 94° C for 1min, annealing at 55° C for 1 min and extension at 72° C for 1

min. The final cycle was followed by a 10 min extension step at 72° C. The concentration of both sets of primers was 0.25 μM and the reaction volume was 50 μl. The amplified cDNA fragments were identified by gel electrophoresis, followed by a confirmation of their specificity by digestion with restriction enzymes (*Pst* I and *Alu* I). The fragments were then subcloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequenced on an Automatic ABI 373 DNA sequencer (Perkin-Elmer, Foster, CA). Sequence analysis was performed with BLASTN program (NCBI, Bethesda, MD).

Northern Blot. To prepare the ^{32}P-labeled antisense RNA probe, the newly-discovered 60-base insertion into NR3 cDNA was subcloned into pGEM-TEasy vector (Promega, Madison, WI). The resulting plasmids were linearized by restriction digestion with Nco-I, and antisense RNA was transcribed with SP6 RNA polymerase (Promega, Madison, WI) in the presence of α^{32}P-UTP (New Life Science Product, Boston, MA).

For analysis, 5 μg of poly(A)$^+$ RNA prepared from diencephalon/telencephalon and cerebellum were separated on 1% agarose-formaldehyde gels and transferred to positively-charged nylon membranes (Ambion, Austin, Texas). The membranes were hybridized overnight at 68°C with the ^{32}P-labeled antisense RNA probe and then washed in 2 X SSC and 0.5% SDS solution three times at room temperature. The final wash was in 0.1 X SSC and 0.5% SDS solution for 60 min at 68°C. To visualize the radiolabeling, the membranes were opposed to Kodak XAR film.

Analysis of Regional Distribution and Development. The relative levels of NR3 variants were compared in multiple brain regions of adult rats. The levels of these variants were also examined in total rat brain tissue at embryonic days E15, E17 and E19, and in the combined rat telencephalon and diencephalon (without the olfactory bulb), cerebellum, and olfactory bulb at postnatal days P0, P3, P5, P7, P10, P14, P20, P25 and P60 (the latter age represented the mature animal). At least three rats were examined for each brain region and developmental age.

The identical amounts (10 ng) of cDNAs reversely transcribed from samples of total RNAs isolated from the above-mentioned tissues were used as templates in quantitative PCR assays. The primers GAAGAAAAGCAGCCACGTTCC (forward) and TCGTT GGTTGTCATGACTCA (reverse) were used to amplify 180-bp (long form) and 120 bp (short form) cDNA fragments. For all samples, twenty eight cycles of amplification were performed with the cycle parameters described above for the PCR procedure used in the cDNA cloning. The concentration of primers was 0.4 μM, and the reaction volume was 50 μl. Our preliminary studies established that, under these conditions, the PCR products of serial dilutions of NR3 short and long form-containing plasmids and the corresponding PCR products of dilutions of cDNA reversely transcribed from the total brain RNA, produced parallel linear dilution curves. Furthermore, comparison of the above-mentioned dilution curves for PCR products of plasmids containing long form and short form of NR3 demonstrated similar efficiencies of amplification for fragments of both forms. Finally, our preliminary studies showed that PCR conditions employed in this study amplify fragments of both short and long NR3 forms below saturation levels for all tissues examined (data not shown). This demonstrates that our RT-PCR protocols were appropriate for quantitative analysis of the levels of the NR3 mRNAs (Wu et al., 1998). Upon completion of amplification, each amplified sample was run in parallel with 50bp DNA ladder (Gibco, Gaithersburg, MD) on a 2% ethidium bromide-stained agarose gel.

The regional levels of NR3 variants in adult rat were estimated visually using the above-mentioned gels containing PCR products of these variants. For developmental studies the quantification of the RT-PCR products obtained at different ages was performed with the Universal Software for Electrophoresis & TLC (Advanced American Biotechnology, Fullerton, CA) using digitized images of the ethidium bromide-stained gels. Series of known amounts of PCR products placed in the same gels served as standards. The final results of quantitative RT-PCR were expressed as moles of amplified transcripts per ng of cDNA reversely transcribed from total tissue RNA.

Southern Blot. PCR reaction products obtained from adult rat brain regions listed above were electrophoresed on 2% agarose gels and blotted onto Hybond-N membranes (Amersham, Buckinghamshire, England). The membranes were hybridized overnight at 68°C with the α^{32}P-labeled antisense RNA probe complimentary to the 60-base insert of the long form, or hybridized at 42°C with an oligonucleotide probe common to both long and short forms. The common probe was complementary to nucleotides 3016-3075 of the original clone and it was labeled at its 3'-end with ^{32}P-dTTP. The membranes were washed as described above for the Northern blot, except that the last wash of the membranes labeled with the oligonucleotide probe was conducted at 42° C. The radiolabeled membranes were opposed to Kodak XAR film.

RESULTS AND DISCUSSION

Isolation and Characterization of NR3 Variants. RT-PCR of the telencephalic poly(A)$^+$ RNA using primers flanking the unique C-terminal region of NR3 (pair 1, Fig. 4A), generated two products. The smaller product contained 213 nucleotides, constituting the 2857 to 3075 region of the previously-described NR3 form. The sequence of the larger product was identical to the smaller one, except for a 60-bp insertion at position 3007. To confirm that the novel insert is associated with a variant of the NR3 subunit mRNA, an additional RT-PCR was performed using the second pair of primers surrounding the fragment of NR3 from TMs III-IV extracellular loop to the C-terminal (pair 2, Fig. 4A). Here also two products were amplified: 800-bp fragment which was identical to the previously-cloned NR3, and 860-bp fragment which had the same nucleotide sequence as the shorter fragment except for the 60-bp insertion (Fig. 4A). Finally, to demonstrate that the insert was not an RT-PCR artifact and that mRNA containing such insert exists *in vivo*, we performed northern blot analysis of poly(A)$^+$ RNA from diencephalon/telencephalon and cerebellum with the probe specific for the insert. The Northern blot identified an

approximately 8 kb transcript in both brain regions (Fig.5). The size of the detected transcript was close to that which would be expected for the NR3 mRNA (Ciabarra et al., 1995; Sucher et al., 1995).

These findings clearly demonstrate the existence of a previously unidentified longer form of mRNA encoding the NR3 subunit of the NMDA receptor and, therefore, this subunit may exist in at least two variants. We propose to designate the newly-discovered long form, NR3-l, and the previously-cloned short form, NR3-s. Based on the fact that NR3-l and NR3-s are identical, with exception of a 60-bp insert, and the observation that alternative splicing is fairly common in ionotropic glutamate receptors (Sprengel and Seeburg, 1995), it is very likely that these two variants are generated by alternative splicing of a single gene transcript.

The deduced amino acid sequence of the insert of NR3-l shows that it consists of 20 amino acid residues at the position of amino-acid 1003 within the intracellular domain of the C-terminal (Fig. 4B). A structural analysis of these 20 amino acid residues revealed two potential sites for phosphorylation. One of these sites may be phosphorylated by PKC and cAMP-dependent protein kinase A, while the other site may be phosphorylated by calmodulin-dependent protein kinase II (Fig.4B). This finding may be significant since a large body of evidence implicates phosphorylation of subunit proteins in regulating major properties of NMDA receptors, such as potentiation and subcellular distribution (Chen and Huang, 1991; Ehlers et al., 1995; Ben-Ari et al., 1992; Markham and Segal, 1992). The presence of the potential phosphorylation sites within the insert also parallels the situation observed in the NR1 subunit where PKC-induced phosphorylation is mainly restricted to four serines located on the C1 splice cassette with the alternative splicings determining the presence or absence of these phosphorylation sites in the resulting form (Tingley et al., 1993). This is postulated to be one of the major mechanisms regulating functional activity of the NR1 subunits (form (Tingley et al., 1993). We hypothesize that the same mechanism

operates in determining the sensitivity to phosphorylation, and thus the functional properties, of the NR3 subunits.

Regional Expression of NR3-l and NR3-s Variants. RT-PCR was used to examine the regional expression of the NR3-l and NR3-s variants in the olfactory bulb, frontal, occipital, entorhinal and piriform cortices, hippocampus, striatum, thalamus, cerebellum and spinal cord of adult rat. Two fragments with the sizes expected to be produced by the NR3-l (180 bp) and NR3-s (120bp) mRNAs were amplified from all tissue samples (Fig. 6A). To confirm that these fragments represent the transcripts of the NR3 forms, Southern blots of these products were performed with the insert-specific probe and the probe to a sequence common for both variants. As predicted, the insert-specific probe hybridized only to the larger fragment while the common probe hybridized to both PCR products (Fig. 6B, C).

While both long and short forms of the NR3 mRNA were present in all the brain regions studied, their relative levels differ from region to region (Fig. 6A). The visual examination of the ethidium bromide gels containing RT-PCR products showed that the olfactory bulb was characterized by relatively close levels of NR3-l and NR3-s variants (Fig. 6A). The short form clearly predominated in the frontal and piriform cortices as well as in the hippocampus and spinal cord (Fig. 6A). In contrast, the long form was predominant in the occipital and entorhinal cortices, thalamus and cerebellum. Moreover, the NR3-s variant was barely detectable in the latter region (Fig. 6A). This indicates that, similar to the NR1 splicing variants, expression of forms of the NR3 mRNA is regulated in a region-specific manner. The regional variations in the relative expression of the long and short NR3 forms may provide for more functionally distinct NMDA receptors in different parts of the brain.

Developmental Expression of NR3 Variants. The prenatal expression of the NR3 variants was examined in the whole rat brain. Both forms were detectable as early as E15 with NR3-s being the predominant form (Fig. 7 insert). By E17, the levels of both forms

increased by approximately 50%. At E19, the levels of the NR3-1 variant were increased further by an additional 25%. In contrast, the NR3-s variant remained at the levels seen at E17 (Fig. 7 insert). Therefore, while the expression of the NR3-1 form in the brain increases steadily throughout the third trimester, the expression of the NR3-s form remains virtually unchanged during the second half of this trimester.

The postnatal development of the NR3 mRNAs was studied in the combined telencephalon and diencephalon (without the olfactory bulb), cerebellum and olfactory bulb (Fig. 7). In the diencephalon/telencephalon of newborn rats, the expression of both NR3 variants was nearly twice as high as in adult animals, with the NR3-s clearly being the predominant form (the levels of NR3-1 were only 50% of the levels of NR3-s). The levels of both variants remained steady throughout the first postnatal week and then began to decline to the adult levels, which were reached for the NR3-1 form by P14 and for the NR3-s form by P25. NR3-s continued to be the predominant variant in the diencephalon/telencephalon of adult animals (Fig. 7). In the cerebellum of newborn rats, the levels of the NR3 mRNAs also were much higher than in adult animals (more than eight times higher for NR3-s and nearly twice as high for NR3-1) with NR3-s being the predominant variant (the levels of NR3-1 were only 35% of the levels of NR3-s). After P7, the expressions of both NR3 variants declined sharply reaching close to the adult levels by P10. The decline was much more significant for NR3-s then for NR3-1, and the latter becomes the predominant form in the cerebellum of adult animals (Fig. 7). In the olfactory bulb of newborn animals, the expression of NR3-s was almost twice as high as that of NR3-1. In addition, the levels of NR3-s were much higher at birth than in adults. The expression of this variant declined steadily within the first two postnatal weeks reaching adult levels at P14. In contrast, the levels of the NR3-1 variant remained virtually unchanged from birth to adulthood. This resulted in similar levels of expression of both NR3 variants in the adult olfactory bulb (Fig. 7).

Our observations confirm the previous developmental studies of the NR3-s mRNA which showed that the highest levels of its expression occur within the late prenatal and early postnatal periods, indicating that the NR3-s subunit may be important for the developmental activities of the NMDA receptors (Ciabarra et al., 1995; Sucher et al., 1995). Our findings also demonstrate that NR3-s is the predominant form during these developmental periods, with NR3-l being expressed at much lower levels, which points to NR3-s as the primary NR3 variant involved in regulation of brain development. NR3-l, however, may also be involved in some developmental actions of the NMDA receptor. This is suggested by its over expression in the diencephalic/telencephalic and cerebellar tissues of newborn animals. It may be significant that, in contrast to other brain regions examined, the olfactory bulb does not display a significant postnatal decline in the NR3-l expression. This may be related to the fact, that the olfactory bulb retains many properties of immature nervous system and undergoes continues reorganization in adult individuals (Farbman, 1994).

The present study demonstrates the existence of a new longer variant of the mRNA encoding the NR3 subunit of the NMDA receptor. This mRNA differs in its regional and developmental expression from that of the previously cloned shorter NR3 mRNA which suggests that the long form of the NR3 subunit brings different properties to the NMDA complexes as compared to the shorter form of this subunit. The unique properties of the newly-described long NR3 subunit are likely related to the presence of two additional phosphorylation sites within its amino acid sequence which affects the regulation of this subunit.

FIGURE LEGENDS

Figure 4. **A.** Alignment of the previously reported sequence of the TM III - C-terminal of the NR3 cDNA (Ciabarra et al., 1995; Sucher et al., 1995) and the large RT/PCR product obtained in the present study (marked by asterisk). The 60-base insertion between the

nucleotides 3006 and 3007 is in bold. The TM III and TM IV are underlined. Primers used for RT-PCR cloning are indicated with arrows. **B**. Deduced amino acid sequence of a portion of the C-terminal of the long NR3 variant. The upper portion depicts the 20 amino acid insertion between the amino acids 1002 and 1003. The potential phosphorylation sites for PKC (Δ), PKA (#) and CaMK-II (§) are indicated. The numbering of the nucleotide and amino acid sequences is according to Ciabarra et al., 1995. The Genebank accession number of the new nucleic acid sequence is AF061945.

Figure 5. Northern blot of the NR3-1 variant from diencephalon/telencephalon (D/T) and cerebellum (CB). Poly(A)$^+$ RNA was loaded on 1% agarose-formaldehyde gel and then transferred to nylon membrane. The blots were visualized by hybridization with insert-specific ^{32}P-antisense RNA probe followed by exposure to Kodak XAR film.

Figure 6. Distribution of the transcripts of the NR3-1 and NR3-s variants in different regions of the central nervous system. 10 ng samples of cDNA reversely transcribed from total RNA from nine regions of the rat brain were amplified, and 15 µl of each amplified sample eletrophoresed on 2% agarose gels as described in the methods. The agarose gels were blotted to a nylon membranes and the specificity of the amplified fragments was checked by hybridization with an insert specific antisense ^{32}P-probe and a ^{32}P-probe common for both long and short NR3 variants. **A.** The agarose gel electrophoresis of RT-PCR products stained with ethidium bromide and illuminated with UV light. Note that two fragments with the size expected to be produced by PCR of the NR3-1 (180 bp; I) and NR3-s (120bp; II) mRNAs were amplified from all tissue samples. This gel demonstrates that the olfactory bulb (OB) has relatively similar levels of both NR3 variants. NR3-s predominates in the frontal cortex (FC), piriform cortex (PC), hippocampus (HP), striatum (ST) and spinal cord (SC). NR3-1 predominates in the occipital cortex (OC), entorhinal cortex (EC), thalamus (TH) and cerebellum (CB). **B.** Hybridization with the insert-specific

probe. Note that this probe hybridized only to the large fragment. **C.** Hybridization with the probe common for both long and short NR3 variants. Note that both long and short fragments were hybridized to this probe.

Figure. 7. Developmental changes in the expression of the NR3-s and NR3-l mRNAs. The transcript levels of the variants were determined by quantitative RT-PCR. The prenatal levels were measured in the whole brain (insert). The postnatal levels were examined in the combined diencephalon and telencephalon (without the olfactory bulb), cerebellum and olfactory bulb. The results were expressed as pmol of RT-PCR product per ng of cDNA reversely transcribed from the total tissue RNA. Each point is an average of three reactions ±SEM.

A.

NR3 2220 GACTGGAAGGTTTCTGATGAATCTTTGGGCCATTTTCTGTATGTTTTGCCTTTC

TM III

2274 TACATACACAGCGAACTTGGCTGCTGTCATGGTAGGTGAGAAGATCTATGAAGAGCTTTC

2334 TGGAATTCATGACCCTAAGCTTCATCATCCTTCTCAAGGCTTCCGCTTTGGAACTGTCCG

2394 GGAAAGCAGTGCTGAAGACTATGTGCGCCAGAGCTTCCCAGAGATGCATGAGTACATGAG

2454 AAGGTACAACGTGCCAGCCACCCCTGATGGAGTGCAGTATCTGAAGAATGATCCAGAGAA

2514 ACTAGACGCCTTCATCATGGACAAAGCCCTTCTGGATTATGAAGTGTCAATAGATGCTGA
*2514 ACTAGACGCCTTCATCATGGACAAAGCCCTTCTGGATTATGAAGTGTCAATAGATGCTGA

pair 2

2574 CTGCAAGCTTCTGACCGTAGGAAAGCCATTTGCCATCGAAGGATATGGCATTGGTCTCCC
*2574 CTGCAAGCTTCTGACCGTAGGAAAGCCATTTGCCATCGAAGGATATGGCATTGGTCTCCC

2634 TCCAAACTCTCCATTGACCTCTAATATATCTGAGCTCATCAGTCAGTACAAGTCTCACGG
*2634 TCCAAACTCTCCATTGACCTCTAATATATCTGAGCTCATCAGTCAGTACAAGTCTCACGG

2694 GTTTATGGATGTGCTCCATGACAAGTGGTACAAGGTGGTTCCCTGCGGAAAGAGAAGCTT
*2694 GTTTATGGATGTGCTCCATGACAAGTGGTACAAGGTGGTTCCCTGCGGAAAGAGAAGCTT

2754 TGCCGTCACTGAGACTTTGCAAATGGGCATCAAGCACTTCTCTGGACTCTTCGTGCTGTT
*2754 TGCCGTCACTGAGACTTTGCAAATGGGCATCAAGCACTTCTCTGGACTCTTCGTGCTGTT

TM IV

2814 GTGCATAGGATTTGGTCTCTCCATCCTGACCACCATTGGTGAACACATAGTGCACAGACT
*2814 GTGCATAGGATTTGGTCTCTCCATCCTGACCACCATTGGTGAACACATAGTGCACAGACT

pair 1

2874 GCTGTTACCACGCATCAAAAACAAATCCAAGCTGCAGTACTGGCTGCACACGAGTCAGAG
*2874 GCTGTTACCACGCATCAAAAACAAATCCAAGCTGCAGTACTGGCTGCACACGAGTCAGAG

pair 1

2934 GTTTCACAGAGCATTAAACACGTCATTCGTAGAAGAAAAGCAGCCACGTTCCAAGACAAA
*2934 GTTTCACAGAGCATTAAACACGTCATTCGTAGAAGAAAAGCAGCCACGTTCCAAGACAAA

2994 ACGTGTGGAGAAG
*2994 ACGTGTGGAGAAG**AGCAGATGGAGAAGATGGACCTGCAAGACAGAAGGGGACTCTG**

3007 AGGTCCAACCTGGGACCCCAGCAGCTCATGGTATGGAATACTT
*3049 **AATTGTCTCTGTTTCCT**AGGTCCAACCTGGGACCCCAGCAGCTCATGGTATGGAATACTT

3050 CCAATCTGAGTCATGACAACCAACGAAAATACATCTTTAATGACGAGGAAGGACAAAACC
*3110 CCAATCTGAGTCATGACAACCAACGAAAATACATCTTTAATGACGAGGAAGGACAAAACC

pair 1

3110 AGCTGGGTACCCAGGCCCACCAGGACATCCCTCTCCCTCAGAGGAGAAGAGAGCTCCCTG
*3170 AGCTGGGTACCCAGGCCCACCAGGACATCCCTCTCCCTCAGAGGAGAAGAGAGCTCCCTG

3170 CCTCACTGACCACCAATGGGAAAGCAGACTCCCTCAATGTAACTCGGAGCTCCGTGATTC
*3230 CCTCACTGACCACCAATGGGAAAGCAGACTCCCTCAATGTAACTCGGAGCTCCGTGATTC

3230 AGGAACTCTCTGAGTTGGAGAAGCAGATCCAAGTGATCCGCCAGGAGCTGCAGTTGGCTG
*3290 AGGAACTCTCTGAGTTGGAGAAGCAGATCCAAGTGATCCGCCAGGAGCTGCAGTTGGCTG

pair 2

3290 TAAGCAGGAAGACAGAGCTGGAGGAGTATCAAAAGACAAATCGGACTTGTGAATCCTAG
*3350 TAAGCAGGAAGACAGAG

pair 2

B.

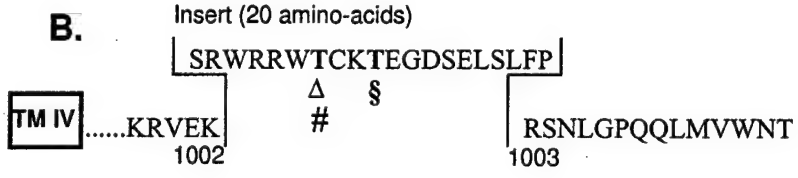

Figure 4

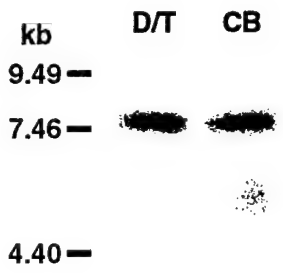

Figure 5

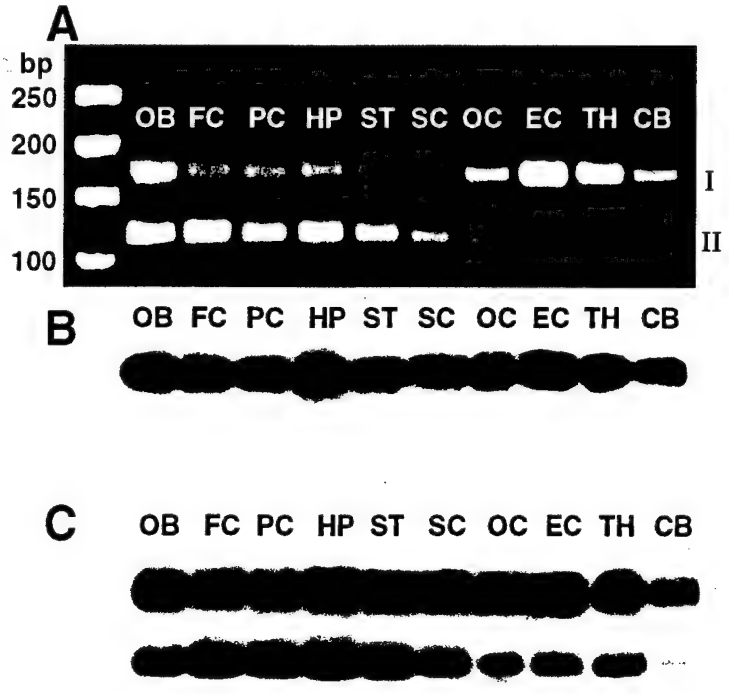
A
bp
250
200
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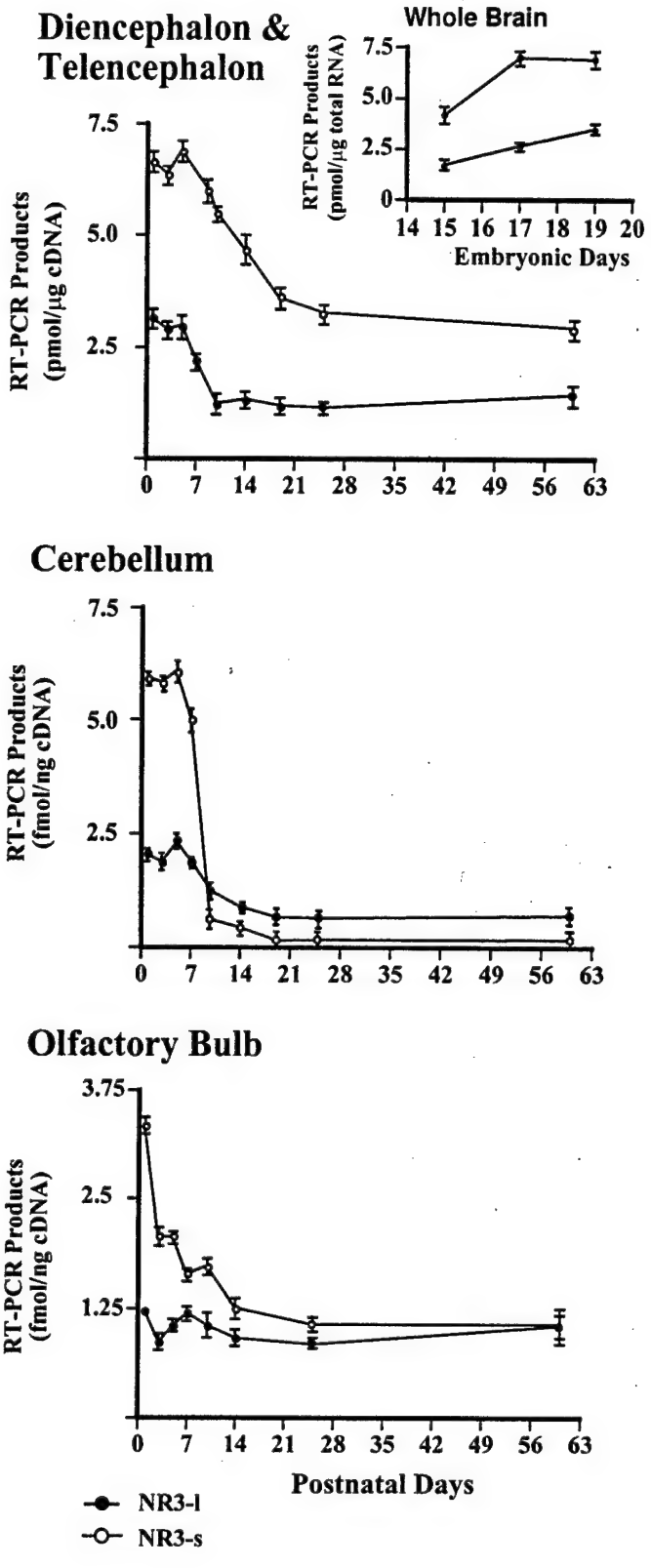
Figure 6


Diencephalon &
Telencephalon
Whole Brain
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(pmol/μg cDNA)
RT-PCR Products
(pmol/μg total RNA)
Embryonic Days
Cerebellum
RT-PCR Products
(fmol/ng cDNA)
Olfactory Bulb
RT-PCR Products
(fmol/ng cDNA)
Postnatal Days
NR3-l
NR3-s

Figure 7

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